

# Examination of cytotoxicity and mutagenicity of AH26 and AH Plus sealers

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## Abstract

**Miletić I, Jukić S, Anić I, Željezić D, Garaj-Vrhovac V, Osmak M.** Examination of cytotoxicity and mutagenicity of AH26 and AH Plus sealers. *International Endodontic Journal*, **36**, 330–335, 2003.

**Aim** To study *in vitro* the cytotoxic and mutagenic effects of AH26 and AH Plus.

**Methodology** Cytotoxic effects on Chinese hamster V79 cells were determined by counting viable cells following incubation with elutions of AH26 and AH Plus. In one set of experiments, the materials were mixed, set for 1 h and then eluted with dimethyl sulphoxide (DMSO) for 1 h, 24 h and 7 days. In the other set, AH26 and AH Plus were mixed and set for 1 h, 24 h and 7 days in physiological saline then crushed and eluted in DMSO for 24 h. The cytotoxic effects of these eluates were evaluated. Three concentrations were chosen to examine the mutagenic effects of AH26 and AH

Plus: 5.57, 16.7 and 55.7  $\mu\text{g mL}^{-1}$ . The structural chromosomal aberration analysis and micronucleus test were performed on human lymphocytes according to standard procedures.

**Results** Dose–response curves of cell survival were obtained. Both materials were shown to be cytotoxic in doses larger than 55.7  $\mu\text{g mL}^{-1}$ , except for AH26, after 7 days setting time. AH Plus was also shown to be toxic in concentrations of 16.7  $\mu\text{g mL}^{-1}$ , except after 7 days setting time. Neither AH26 nor AH Plus induced a significant increase of chromosomal aberrations or micronuclei induction at any setting time or concentration.

**Conclusion** There was no mutagenicity found for AH26 and AH Plus on human lymphocytes in highly controlled conditions *in vitro*.

**Keywords:** AH26, AH Plus, chromosomal aberrations, human lymphocytes, micronucleus, V79.

Received 14 June 2002; accepted 22 November 2002

## Introduction

The purpose of root canal treatment is to eliminate infection in the root canal and to fill the root canal space. Various commercial sealers have been developed and used for this purpose. One of them, AH26 sealer (Dentsply, DeTrey, Konstanz, Germany), is frequently used because of its excellent sealing ability (Wu *et al.* 1995, Miletić *et al.* 1999). It has been demonstrated, however, that the sealer was cytotoxic during setting (Gerosa *et al.* 1995) which can be, to some extent, explained by the release of formaldehyde (Spangberg *et al.* 1993, Koch 1999). A modified version of the material AH Plus (Dentsply) was subsequently developed. According to the manufacturer, AH

Plus has better physical and clinical properties than AH26 and the formulation no longer releases formaldehyde.

Root filling materials are usually in close contact with living tissues. Thus, the biological properties of these materials are important as cytotoxic materials can damage periapical tissues, and material with mutagenic potential can induce DNA mutations, possibly causing malignant transformation of the cells (Bertram 2001). Various tests have been developed for determination of mutagenicity. The most commonly used and simplest is the Ames test (Lewis & Chestner 1981). However, the positive results of the Ames test alone are not sufficient to estimate the carcinogenic risk to a human population (Cross *et al.* 1983); rather, these are used to detect potentially dangerous chemicals (Lewis & Chestner 1981). The genotoxicity of dental material should also be investigated using other tests, such as the V79/hprt

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mutation assay, the micronucleus test and the chromosomal aberration assay (Fenech & Morley 1985, Garaj-Vrhovac & Željezić 2001). The mutagenic effects of AH26 have been determined on rodent cells *in vitro* using the mutation assay with the mutagenic effect being dependent on the period after mixing (Schweikl *et al.* 1995).

Data on the mutagenic effects of AH26 and AH Plus on human cells is inconclusive. Therefore, the aim of this study was to determine the cytotoxic and mutagenic effects of AH26 and AH Plus *in vitro* using a structural chromosomal aberration analysis and micronucleus test.

## Materials and methods

### AH Plus and AH26 sealers

In the present study, two materials were used: AH26 silver free (Dentsply, DeTrey) and AH Plus (Dentsply, DeTrey). The sealers were mixed according to the manufacturer's instructions in aseptic conditions. In one group of experiments, the mixed material was set for 1 h and crushed; then 0.1 g of the material was eluted with 2 mL of dimethyl sulphoxide (DMSO) for 1 h, 24 h and 7 days. These extracts were serially diluted and used for further examination to final concentrations of 1.67, 5.57, 16.7, 55.7 and 167 g mL<sup>-1</sup>. These were chosen after the concentration of 167 g mL<sup>-1</sup> demonstrated cytotoxicity in the pilot study, so a range of smaller concentration were used to determine doses not causing a significant cytotoxic effect. In the other group of experiments, the material was set for 1 h, 24 h or 7 days in a physiological saline and eluted using the same protocol. The control samples were treated with DMSO diluted in the same way and added to the culture samples.

### Cell cultures

Chinese hamster V79 cells were grown as a monolayer culture in Dulbecco's modified essential medium, which was supplemented with 10% foetal calf serum and antibiotics, in a humid atmosphere containing 5% CO<sub>2</sub> (Atcc, Global Bioresource Centre, Manassas, VA, USA).

Human lymphocytes were kept at 37°C in the F-10 medium (IAEA 1986) in the presence of 0.5 mL phytohaemagglutinine and with 10% of newborn calf serum (Biological Industries, Kibutz, Beit Haemek, Israel).

### Cytotoxicity assay

Chinese hamster V79 cells were plated in 2 mL growth medium in 24-well plates (7.5 × 10<sup>3</sup> cells per plate). The

cells were incubated for 72 h. Thereafter, they were trypsinized and counted. In parallel samples, the number of viable cells was determined using nigrosin dye. The cytotoxicity was quantified by comparing the number of viable cells in treated samples with the corresponding number of viable cells in the control samples. Each experiment was repeated three times.

### Mutagenicity assay

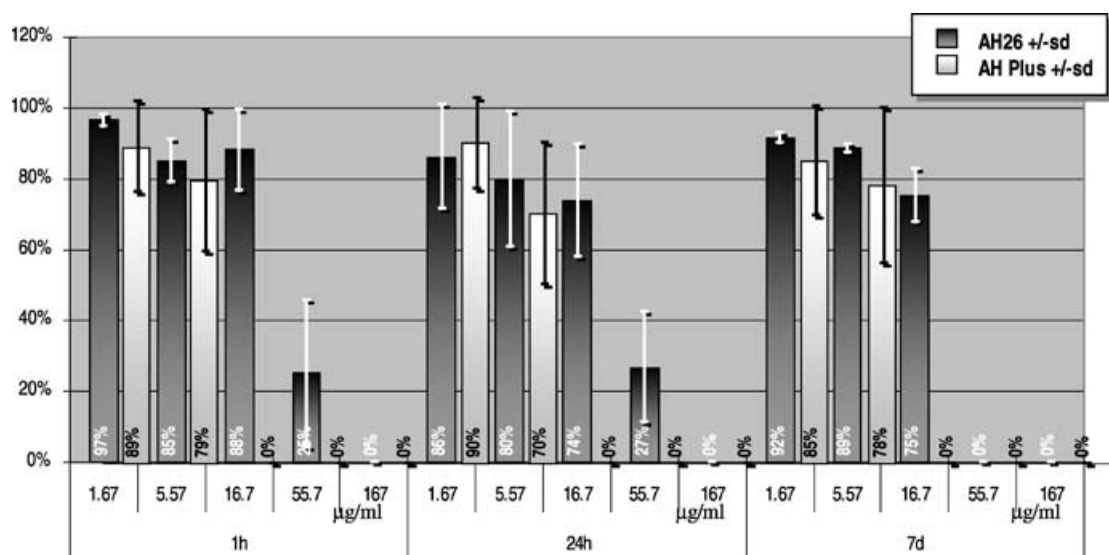
To determine the possible mutagenic effect of AH26 and AH Plus, two standard cytogenetic methods were used: the structural chromosomal aberration analysis and the micronucleus test. Both methods were performed on the cultures of human peripheral blood lymphocytes. At the beginning of the procedure, lymphocytes were treated with 5.57, 16.7 and 55.7 g mL<sup>-1</sup> of AH26 and AH Plus, all in the range of cytotoxic concentrations (Figs 1 and 2). The control lymphocytes were treated with DMSO diluted in the same way. For the structural chromosome aberration analysis, the cultures were harvested 48 h after their initiation. Three hours prior to harvesting, 0.2 g mL<sup>-1</sup> of colchicine was added. After the slide preparation, 200 metaphases per sample were analysed. For the micronucleus test, 44 h after the culture initiation, 3 g mL<sup>-1</sup> of cytochalasin B (Sigma, St. Louis, USA) was added. Total time of the cultivation was 72 h. After the slide preparation, 500 binucleated lymphocytes per sample were analysed. The number of chromatid and chromosome breaks and acentric fragments, as well as number of micronuclei was recorded for each sample.

### Statistical analysis

Statistical analysis of cytotoxicity data was performed using the *F*-test for ratios of the cell number in experimental and control samples, as data had shown no significant inhomogeneity of variances. As mutagenic data consisted only of proportion of aberrations and micronuclei, these were evaluated by testing differences between proportions in experimental versus control samples.

## Results

As shown in Fig. 1, both sealers showed strong cytotoxic effects in concentrations of 55.7 and 167 g mL<sup>-1</sup> for all three periods, while for the concentration of 16.7 g mL<sup>-1</sup>, AH Plus had a significantly stronger cytotoxic effect than AH26 (*F*-test, *P* < 0.05 for all three periods).



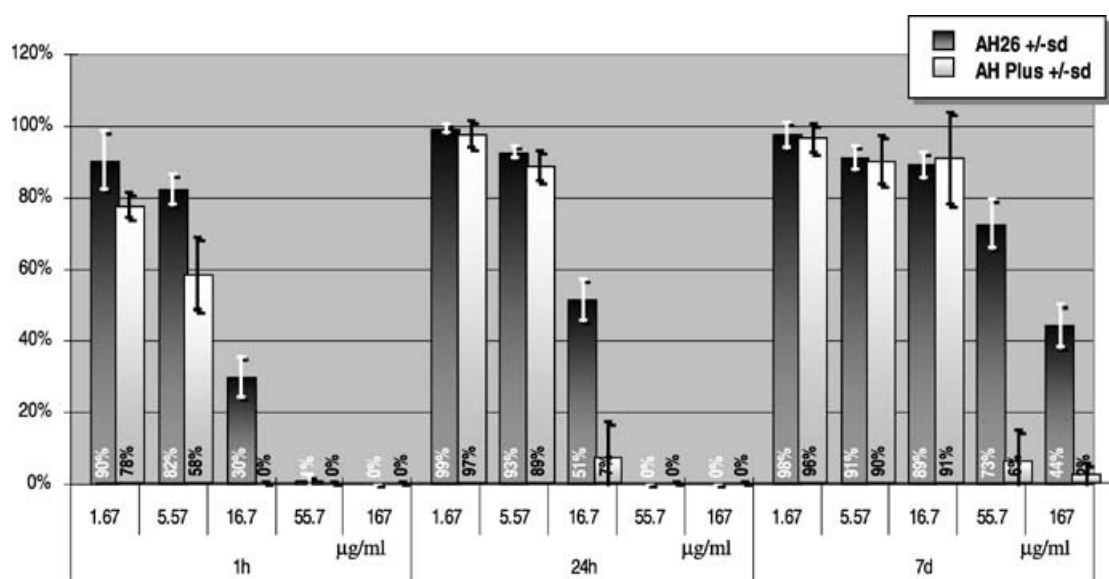
**Figure 1** Cytotoxicity of DMSO eluates of mixed AH26 and AH Plus after indicated elution periods.

Response curves were similar in samples eluted for different periods of time (*F*-test,  $P > 0.05$  for both materials and concentrations).

The cytotoxic effects of set material for different periods of time are shown in Fig. 2. Again the dose-response curves of cell survival were obtained. AH Plus was again significantly (*F*-test,  $P < 0.05$ ) more cytotoxic than AH26 in concentrations of  $16.7 \text{ g mL}^{-1}$ , for 1 and 24 h setting times and also at 55.7 and  $167 \text{ g mL}^{-1}$  for the 7-day period. Further, the cytotoxic effect was shown

to be dependent on the setting time. For AH26, reduced cytotoxicity was found for samples set for 7 days, when compared to 24 and 1 h setting times, for concentrations of  $16.7$ ,  $55.7$  and  $167 \text{ g mL}^{-1}$  ( $P < 0.05$  for 24 h vs. 7 days comparison, as well as for 7 days vs. 1 h comparison). The cytotoxicity of AH Plus was reduced only after 7 days for the concentration of  $16.7 \text{ g mL}^{-1}$ .

According to these results, the concentration of  $5.57$ ,  $16.7$  and  $55.7 \text{ g mL}^{-1}$  of AH26 and AH Plus were used for determination of mutagenic potential. Table 1 shows



**Figure 2** Cytotoxicity of DMSO eluates of mixed AH26 and AH Plus after setting in saline solution.

**Table 1** Results of the structural chromosomal aberration analysis and micronucleus test for AH26 and AH Plus with different concentrations and setting times (1 h, 24 h and 7 days)

| Agents                             |        | Chromosomal aberrations |    |    | Micronucleus test       |                        |
|------------------------------------|--------|-------------------------|----|----|-------------------------|------------------------|
|                                    |        | L1                      | L2 | Ac | Total no. per 500 cells | Distribution (MN/cell) |
| AH Plus 5.57 $\mu\text{g mL}^{-1}$ | DMSO   | 1                       | 1  | 0  | 3                       | 3 × 1                  |
|                                    | 1 h    | 1                       | 1  | 0  | 5                       | 3 × 1                  |
|                                    |        |                         |    |    |                         | 1 × 2                  |
|                                    | 24 h   | 1                       | 1  | 1  | 3                       | 3 × 1                  |
|                                    | 7 days | 1                       | 0  | 1  | 3                       | 3 × 1                  |
| AH Plus 16.7 $\mu\text{g mL}^{-1}$ | DMSO   | 1                       | 0  | 0  | 6                       | 6 × 1                  |
|                                    | 1 h    | 2                       | 0  | 0  | 8                       | 8 × 1                  |
|                                    | 24 h   | 1                       | 2  | 0  | 8                       | 8 × 1                  |
|                                    | 7 days | 2                       | 2  | 1  | 12                      | 10 × 1                 |
|                                    |        |                         |    |    |                         | 1 × 2                  |
| AH Plus 55.7 $\mu\text{g mL}^{-1}$ | DMSO   | 1                       | 0  | 1  | 6                       | 6 × 1                  |
|                                    | 1 h    | 2                       | 3  | 0  | 7                       | 7 × 1                  |
|                                    | 24 h   | 1                       | 0  | 1  | 5                       | 5 × 1                  |
|                                    | 7 days | 2                       | 1  | 0  | 6                       | 6 × 1                  |
| AH26 5.57 $\mu\text{g mL}^{-1}$    | DMSO   | 1                       | 0  | 0  | 3                       | 3 × 1                  |
|                                    | 1 h    | 0                       | 0  | 0  | 4                       | 4 × 1                  |
|                                    | 24 h   | 0                       | 1  | 0  | 3                       | 3 × 1                  |
|                                    | 7 days | 1                       | 1  | 0  | 4                       | 4 × 1                  |
| AH26 16.7 $\mu\text{g mL}^{-1}$    | DMSO   | 1                       | 0  | 0  | 4                       | 4 × 1                  |
|                                    | 1 h    | 1                       | 0  | 0  | 4                       | 4 × 1                  |
|                                    | 24 h   | 0                       | 1  | 0  | 5                       | 5 × 1                  |
|                                    | 7 days | 1                       | 0  | 1  | 3                       | 3 × 1                  |
| AH 26 55.7 $\mu\text{g mL}^{-1}$   | DMSO   | 1                       | 0  | 1  | 6                       | 6 × 1                  |
|                                    | 1 h    | 2                       | 2  | 2  | 12                      | 12 × 1                 |
|                                    | 24 h   | 2                       | 0  | 1  | 8                       | 6 × 1                  |
|                                    |        |                         |    |    |                         | 1 × 2                  |
|                                    | 7 days | 2                       | 2  | 1  | 10                      | 10 × 1                 |

L1: chromatid break; L2: chromosome break; Ac: acentric fragment; MN: micronucleus.

the results of the structural chromosomal aberration analysis and the micronucleus test. In the cultures treated with AH Plus, chromatid and chromosome breaks and acentric fragments were found. However, there was no significant difference in their numbers between control cells treated with corresponding dilution of DMSO and lymphocytes treated with AH26 and AH Plus. Also, no significant difference was found regarding the setting time or elution time of the AH Plus (1 h, 24 h and 7 days). Lymphocytes treated with AH26 did not show any significant increase in the number of structural chromosomal aberrations.

The micronucleus test showed results similar to those of the structural aberration analysis. There were no statistical differences between treated and control samples. Only in the blood samples treated with 16.7  $\text{g mL}^{-1}$  AH26 and 16.7  $\text{g mL}^{-1}$  AH Plus that were obtained by 7 days elution in DMSO, there was a slight but insignifi-

cant increase in the number of aberrations and micronuclei. In the samples treated with 55.7  $\text{g mL}^{-1}$  AH26 obtained by 1 h and 7 days setting period, a statistically insignificant increase in the frequency of micronuclei was found.

## Discussion

In this study, the cytotoxicity and mutagenicity of two root canal sealers, AH26 and AH Plus, were investigated *in vitro*. The cytotoxicity was dependent on concentration, setting time and the sealer used. Both materials exhibited reduced cytotoxicity when set for longer and did not have increased toxicity when eluted for a longer period. AH Plus showed significantly stronger cytotoxicity than AH26, both initially and after longer setting intervals. Such results would imply that the substances responsible for cytotoxicity of AH26 are

released primarily during the first 24 h of setting. Although AH Plus set for 1 h did not increase cytotoxicity in prolonged elution, it still exhibited strong cytotoxicity after 7 days. Schweikl *et al.* (1995) reported that eluates of mixed AH 26 set for 7 days were weakly toxic compared with freshly mixed material. Cytotoxicity of AH Plus found in this study is in accordance with findings of Schweikl & Schmalz (2000) who reported reduction of cell survival rates to 50% after exposure to 30 mg mL<sup>-1</sup> of this material. They claimed that eluting substance had a significant influence on cytotoxicity of materials tested, with the greatest toxic effect occurring with AH Plus before elution, followed by AH Plus eluted in DMSO; elutes of AH Plus in 0.9% sodium chloride were less toxic. This study has attempted to avoid the possible influence of DMSO itself by using the proportion of viable cells in the eluate to the number of viable cells in DMSO to assess cytotoxicity.

In the present study, three concentrations of AH26 and AH Plus (which have shown a range from mild to strong cytotoxic effect) were used to examine the mutagenic effect following the setting periods of 1 h, 24 h and 7 days. The results show that neither lower nor higher concentrations of these materials, and neither shorter nor longer setting periods increased the number of chromosomal aberrations or micronucleus induction.

Published data on the mutagenic effects of AH26 and AH Plus are conflicting. Schweikl *et al.* (1995) found that AH26 was mutagenic in the rodent cells mutation assay; the mutagenic effect of AH26 decreased with setting time, but continued for 7 days. Schweikl & Schmalz (2000), using the induction of micronuclei in Chinese hamster V79 as the end point of their study, observed mutagenicity of freshly mixed AH Plus eluted with DMSO, but no mutagenicity after 24 h setting time. Schweikl *et al.* (1995) have shown that the *in vitro* mutagenicity of AH26 is based mainly on an epoxy resin, bisphenol A diglycidylether. This component is also a 'precursor' of AH Plus (Schweikl & Schmalz 2000), but they did not find any mutagenic activity of mixed AH Plus after a setting time of 24 h.

In a previous study, both AH26 and AH Plus were mutagenic when assessed by the Ames test, but the mutagenic potential of AH Plus was shown to be weaker than that of AH26 (Jukić *et al.* 2000). On the other hand, Leyhausen *et al.* (1999) found no mutagenic potential with the Ames test using same *Salmonella* strains. Thus, research data about mutagenic potential of AH26 and AH Plus obtained *in vitro* are at variance, even when they were obtained using similar test systems. However, the most important mutagenicity data, obtained with

human cells, are missing. In the present study, the mutagenic effects of root canal sealers AH26 and AH Plus on human lymphocytes were examined and compared; there was no mutagenicity either in chromosomal aberration analysis or in the micronuclei induction for the examined eluates. The finding that AH26 and AH Plus may not be mutagenic for human lymphocytes in highly controlled conditions *in vitro*, may have important clinical implications. Although the results indicate inherent mutagenic potential of the materials tested, a short-term mutagenicity assay cannot predict mutagenic changes that can take many years or several generations to appear and the tested doses can be significantly different to the dose actually delivered.

## Conclusion

AH26 and AH Plus were shown to be cytotoxic in larger doses, while there was no mutagenicity found in both materials on human lymphocytes in highly controlled conditions *in vitro*.

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